

REVIEW ARTICLE

Emergent mechanistic diversity of enzyme-catalysed β -diketone cleavage

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The enzymatic cleavage of C–C bonds in β -diketones is, comparatively, a little studied biochemical process, but one that has important relevance to human metabolism, bioremediation and preparative biocatalysis. In recent studies, four types of enzymes have come to light that cleave C–C bonds in the β -diketone functionality using different chemical mechanisms. OPH [oxidized poly(vinyl alcohol) hydrolase from *Pseudomonas* sp. strain VM15C], which cleaves nonane-4,6-dione to butyrate and pentan-2-one is a serine-triad hydrolase. Dke1 (diketone-cleaving enzyme from *Acinetobacter johnsonii*) is a dioxygenase, cleaving acetylacetone to methylglyoxal and acetate. Fumarylacetoacetate hydrolase cleaves fumarylacetoacetate to fumarate and aceto-

acetate using a water molecule, activated by a catalytic His/Asp dyad, aided by a calcium ion that both chelates the enol acid form of the substrate and indirectly positions the water for nucleophilic attack at a carbonyl group. 6-Oxocamphor hydrolase cleaves non-enolizable cyclic β -diketones and is a homologue of the crotonase superfamily, employing a catalytic His/Asp dyad to activate a water molecule for nucleophilic attack at a carbonyl group on one prochiral face of the diketone substrate, effecting desymmetrizations of symmetrical substrates.

Key words: catalytic triad, crotonase, β -diketone, dioxygenase, hydrolase, polyketide.

INTRODUCTION

β -Diketones (Figure 1) are an important class of organic compounds frequently encountered in synthetic chemistry [1–3], where, in addition to their established role in the synthesis of *N*-heterocycles [4], they have been used as ligands for the co-ordination of transition metals [5] and have also been investigated for use as potential antiviral agents [6]. β -Diketones are also encountered in Nature as both metabolic intermediates in the microbial metabolism of aromatics and terpenes and also as anthropogenic environmental contaminants (see below). As a result of their ubiquity, the biological transformation of these compounds has recently aroused interest [7,8]. The present review will focus on the mechanistic diversity that has recently been observed in those enzymes that catalyse the cleavage of a C–C bond between the carbonyl groups of β -diketones bearing substituents R^1 and R^2 , where these substituents are not a hydroxy group or an O-alkyl or S-linked thioester of CoA (Figure 1). Hence, no further reference will be made to additional diversity encountered in the cleavage of those β -dicarbonyl compounds. ' β -Diketone hydrolases' as such are nominally described by the Enzyme Commission designation EC 3.7.1.- {“Hydrolases (3) acting on C–C bonds (7) in ketonic substances (1)”} (<http://www.chem.qmul.ac.uk/iubmb/enzyme/>), although, as has been observed, such distinctions with a basis primarily in observed reaction chemistry are often at odds with sequence-, structure- or mechanism-based descriptors when the nature of the enzyme activity has been revealed [10].

ENZYMES THAT CLEAVE β -DIKETONES

There have been reports of the enzymatic cleavage of β -diketones in the microbiological literature for a number of years, although the preliminary nature of the studies meant that little more than

cursory investigations of mechanism were presented. An initial examination suggests that two types of substrates are cleaved: β -diketo acids, where R^2 is a carboxylate in an α or β position to one of the diketone carbonyl groups, and 'neutral' β -diketones, where R^1 and R^2 are alkyl groups that form part of larger aliphatic or alicyclic systems (Figure 1).

C–C BOND CLEAVAGE IN β -DIKETO ACIDS

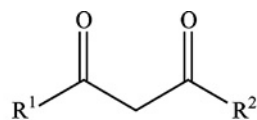
The cleavage of β -diketo acids is common in Nature and can be observed in the eukaryotic metabolism of amino acids and degradation of aromatic compounds by prokaryotes. The enzymatic hydrolysis of acetylpyruvate (**1**) to acetate (**2**) and pyruvate (**3**) (Scheme 1) had been described as part of the degradation of catechol by a *Pseudomonas* sp. in the early 1960s [11], and these reports were closely followed by the work of Davey and Ribbons [12], who isolated and partially characterized an APH (acetylpyruvate hydrolase) from *Pseudomonas putida* when this organism was grown on resorcinol as sole carbon source. That work was recently revisited with the use of elegant NMR experiments that suggested that the actual substrate for the enzymatic reaction was the carboxylate 2-enol form [13]. Activity was at this time attributed to base-catalysed activation of a water molecule for nucleophilic attack at the four-carbon carbonyl group, with a bivalent Mg^{2+} chelating the enol carboxylate (Figure 2).

Mechanism of FAH (fumarylacetoacetate hydrolase, EC 3.7.1.2)

The best evidence of such a mechanism for the cleavage of C–C bonds in β -diketo acids comes from studies on FAH. The hydrolysis of fumarylacetoacetate (**4**) to fumarate (**5**) and acetoacetate (**6**) (Scheme 2) constitutes a late step in the mammalian metabolism of tyrosine, and disruptions to the gene encoding FAH

Abbreviations used: APH, acetylpyruvate hydrolase; Dke, diketone-cleaving enzyme from *Acinetobacter johnsonii*; FAH, fumarylacetoacetate hydrolase; FPH, fumarylpyruvate hydrolase; HMPOBA, 4-(hydroxymethylphosphinoyl)-3-oxobutanoic acid; MPH, maleylpyruvate hydrolase; OCH, 6-oxocamphor hydrolase from *Rhodococcus* sp.; OPH, oxidized PVA [poly(vinyl alcohol)] hydrolase from *Pseudomonas* sp.; PDH, pentane-2,4-dione hydrolase; T4HN, 1,3,6,8-tetrahydroxynaphthalene.

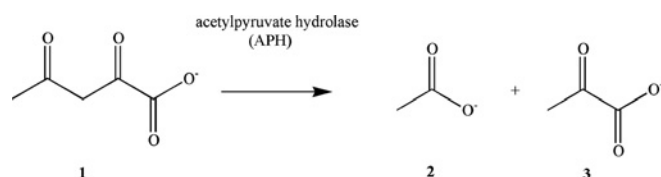
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$R^1, R^2 = \text{Alkyl}; \text{'neutral' } \beta\text{-diketone}$

$R^1 = \text{alkyl}; R^2 = \text{CO}_2^-, \text{CH}_2\text{CO}_2^-; \beta\text{-diketoacid}$

Figure 1 Two types of β -diketone cleaved by enzymes



Scheme 1 Hydrolysis of acetylpyruvate by APH

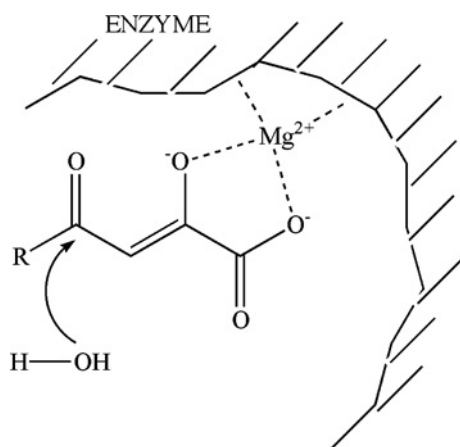
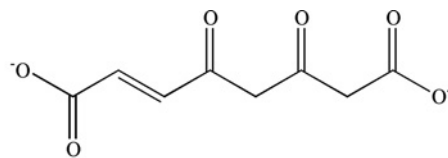


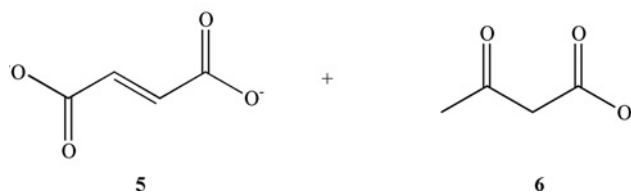
Figure 2 Suggested mechanism for the action of APH from *Pseudomonas putida*

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in humans have been shown to be the most significant factor in Type 1 hereditary tyrosinaemia [14]. After early reports of FAH activity in both human liver preparations [15] and bacteria [16], an enzyme was purified from ox liver that was observed to catalyse the cleavage of not only fumarylacetoacetate, but also acetylpyruvate *in vitro* [17]. The enzyme was later described as hydrolysing a series of additional β -diketones, including six other 2,4-diketo acids and three 3,5-diketo acids of those tested [18]. However, any suggestions as to the mechanism of FAH-catalysed C–C bond cleavage were largely speculative until the structure of mouse FAH was solved in 1999 by Timm et al. [19]. Mouse FAH, which was shown not to have any close sequence-related homologues, was revealed to be a dimeric enzyme of subunit molecular mass 46 kDa (Figure 3). FAH was a calcium-containing α/β -hydrolase of novel fold and containing an N-terminal domain of 120 amino acids and a C-terminal domain of 330 amino acids



Fumarylacetoacetate hydrolase (FAH)



Scheme 2 Reaction catalysed by FAH

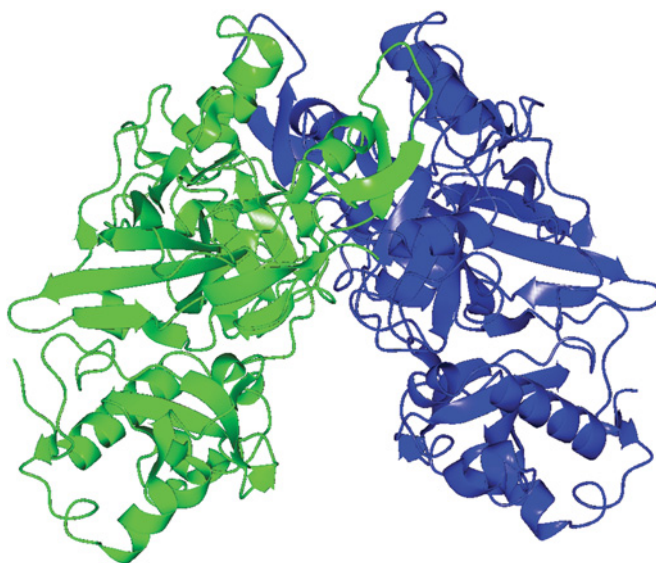
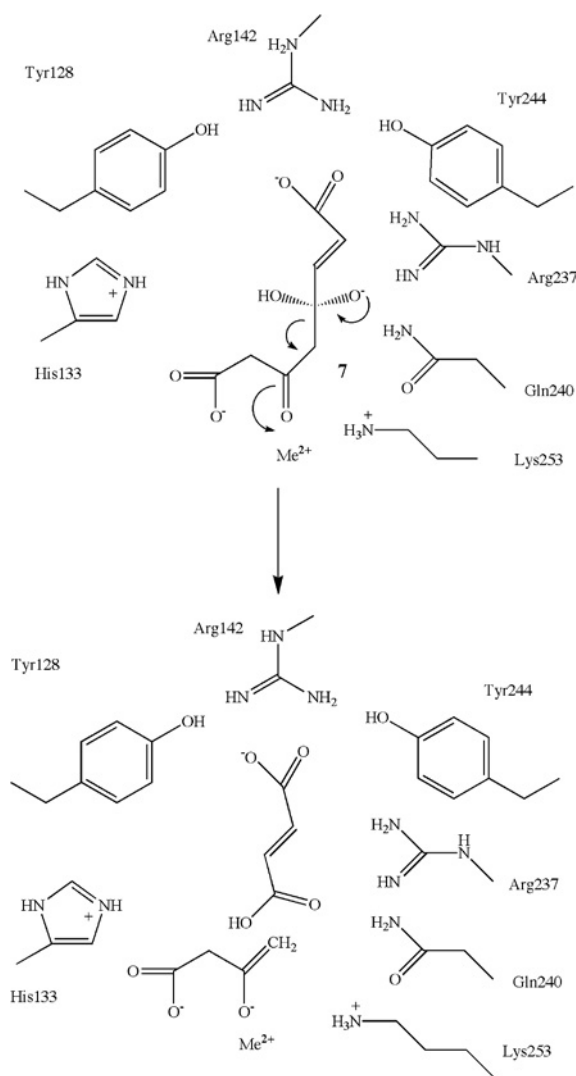


Figure 3 Structure of FAH – a dimer of two 46 kDa subunits

arranged in an unusual mixed β -sandwich roll. The active site was located near the dimer interface, and it was observed to bind the products of fumarylacetoacetate hydrolysis, namely fumarate and acetoacetate.

Fumarate was bound near to the entrance to the active site; acetoacetate was bound to a calcium ion in close proximity to His¹³³, which appeared to form a dyad with Glu³⁶⁴. The relative positions of the active-site dyad and the acetoacetate residue were suggested to be consistent with a mechanism whereby the enolate form of fumarylacetoacetate would be co-ordinated by the bivalent calcium ion (indicated by 'Me²⁺' in Scheme 3), holding the substrate in place for nucleophilic attack at the electrophilic carbon atom of the target carbonyl group. The hydroxide nucleophile would be generated by general base-catalysed activation of a water molecule by His¹³³, activated in turn by Glu³⁶⁴. The Ca²⁺ ion was also observed to position the water for nucleophilic attack



Scheme 3 Mechanism proposed for FAH-catalysed cleavage of fumarylacetoacetate

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indirectly, as it was co-ordinated to Glu¹⁹⁹, which, in turn, was H-bonded to the water that would furnish the nucleophile. Initial nucleophilic attack of hydroxide would generate a tetrahedral alkoxide (7) that could be stabilized by an oxyanion hole formed by the side chains of residues Arg²³⁷, Gln²⁴⁰ and Lys²⁵³. After C–C bond cleavage, the consequent acetoacetate carbanion would be quenched by a proton from Lys²⁵³ (Scheme 3). The need for co-ordination of the substrate to the calcium ion was thought to be indicative of the relative inactivity of the C–C bond to be cleaved when compared with the C–heteroatom bonds hydrolysed by classical serine-catalytic-triad enzymes such as lipases and proteases.

The mechanism proposed for FAH was reinforced by the solution of the FAH structure in complex with a phosphorus analogue of a putative alkoxide transition state, HMPOBA [4-(hydroxymethylphosphinoyl)-3-oxobutanoic acid, **8**] [20] (Figure 4). In addition to the calcium ion, a magnesium ion was identified which appeared to stabilize the β -roll that constituted the active site of FAH. Comparison of the HMPOBA-bound FAH structure and the native and product-bound structures revealed some flexibility of

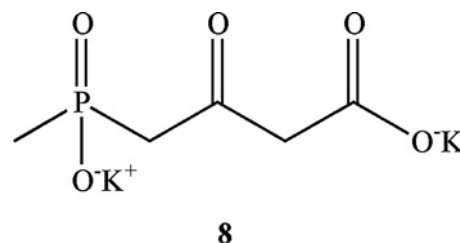


Figure 4 Structure of HMPOBA, an alkoxide transition-state analogue for the inhibition of FAH

active-site-residue side chains, particularly of those thought to form the oxyanion hole, consistent with a change in active-site topology on substrate cleavage.

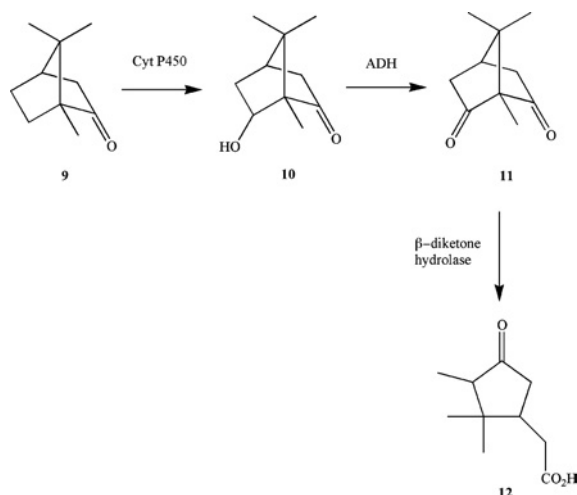
β -Diketone hydrolase activities analogous to that of FAH have been reported in bacteria metabolizing gentisate. Both FPH (fumarylpyruvate hydrolase) and constitutive [MPH I (maleylpyruvate hydrolase I)] and an inducible (MPH II) activities were identified in a strain of *Pseudomonas alcaligenes* [21,22]. Each enzyme was found to be specific for its substrate, FPH and MPH enzymes catalysing the formation of fumarate and pyruvate, and maleate and pyruvate, respectively [23]. A gene encoding FPH has recently been cloned from *Ralstonia* sp. strain U2 [24] and shown to encode a protein of 195 amino acids of approx. 21 kDa molecular mass. This enzyme exhibits approx. 40 % sequence identity with the fumarylacetoacetate hydrolase from *Xanthomonas campestris*, an FAH homologue of only 237 amino acids, which appears to lack the large N-terminal domain of the mouse enzyme. In the absence of structural and mechanistic information, it is difficult to be certain of conserved mechanism of C–C bond cleavage in these bacterial enzymes, but the apparent conservation of some key residues, including the Arg²³⁷ and Gln²⁴⁰, which form a proposed oxyanion hole, is perhaps suggestive of some similarity.

C–C BOND CLEAVAGE IN 'NEUTRAL' β -DIKETONES (R¹ AND R² = ALKYL)

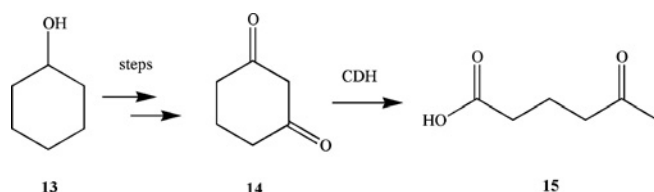
One of the earliest inferences of a neutral β -diketone-cleaving enzyme was made in 1966 by Chapman et al. [25] who, when performing metabolite extraction studies on the mother liquor of fermentations of *Corynebacterium* strain T1 grown on (1R)-(+)-camphor (**9**) as sole carbon source, noted that the symmetrical diketone 6-oxocamphor (**11**, bornane-2,6-dione), appeared to be cleaved to α -campholinic acid (**12**) by a biocatalytic process that yielded an optically active product (Scheme 4). No further biochemical characterization of this activity was presented at that time, but we shall return to the enzyme responsible for the transformation in much greater detail later.

Cyclohexane-1,3-dione (**14**) was reported to be hydrolysed to 5-oxohexanoic ('5-oxocaproic acid', **15**) by cell extracts of a *Pseudomonas* strain grown under anaerobic conditions on cyclohexanol (**13**) as sole carbon source (Scheme 5) [26]. The enzyme, named cyclohexane-1,3-dione hydrolase (EC 3.7.1.10) was not isolated, but kinetic constants for impure preparations were calculated and the enzyme shown not to accept cyclohexane-1,2-dione or cyclohexane-1,4-dione as substrates. The failure to stimulate activity by the addition of CoA was taken as evidence that the enzymatic cleavage of the C–C bond was not performed by a thiolase.

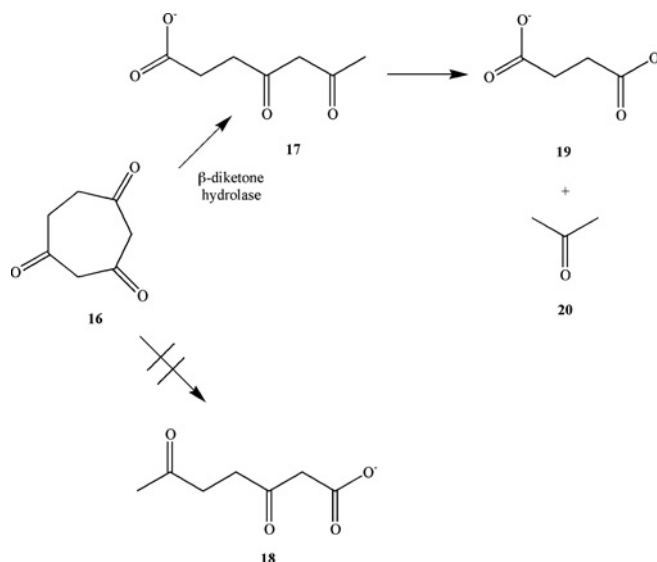
Two β -diketone-cleavage enzymes were reported to be active in the metabolism of atropine by *Pseudomonas* sp. AT3 (Scheme 6) [27]. An induced hydrolase was found to act regiospecifically on



Scheme 4 Metabolism of (1*R*)-(+)-camphor by *Corynebacterium* T1 (= *Rhodococcus* sp. NCIMB 9784)



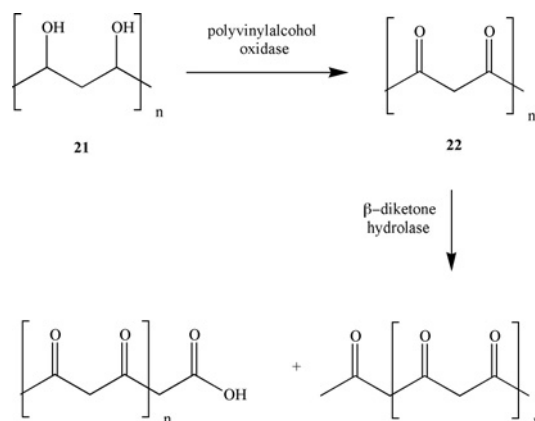
Scheme 5 Cleavage of cyclohexane-1,3-dione by cyclohexane-1,3-dione hydrolase (CDH) from a *Pseudomonas* sp. as part of the anaerobic degradation of cyclohexanol



Scheme 6 Hydrolysis of cycloheptane-1,3,5-trione by a β -diketone hydrolase from *Pseudomonas* strain AT3 involved in atropine degradation

cycloheptane-1,3,5-trione (**16**), cleaving one β -diketone regioselectively to yield a product, **17**, but not the alternative product, **18**. The product **17**, itself a β -diketo acid, was cleaved by a β -diketone hydrolase to yield succinate (**19**) and acetone (**20**).

A β -diketone hydrolase was implicated in the degradation of the polymeric plastic material PVA [poly(vinyl alcohol), **21**]



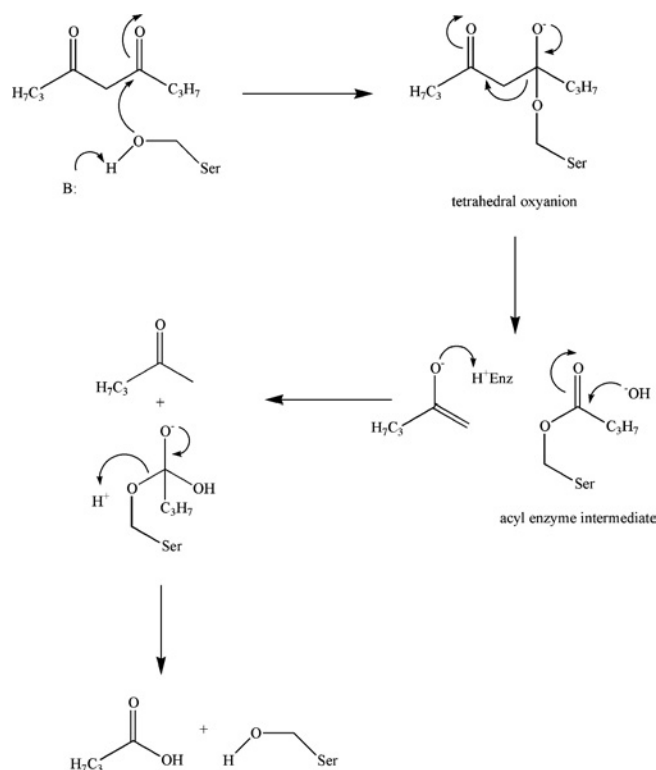
Scheme 7 Degradation of PVA by *Pseudomonas* spp.

(Scheme 7). A strain of *Pseudomonas* was reported by Sakai et al. [28–31] to degrade PVA first by the action of a dehydrogenase, to yield poly(vinyl ketone) (**22**), which was subsequently cleaved by a hydrolase enzyme to yield products with either methyl ketone or carboxylate termini. The activity was tested *in vitro* using nonane-4,6-dione as test substrate, and pentan-2-one and butyrate were the consequent products. The enzyme was also shown to cleave a variety of related alkyl diketones and 1-phenylpentane-2,4-dione, but not cyclic β -diketones such as cyclohexane-1,3-dione. Few biochemical studies were performed with this system, but an analogous enzyme, PDH (pentane-2,4-dione hydrolase), was reported by Kawagoshi and Fujita in 1998 [32]. Isolated from the PVA-degrading strain *Pseudomonas vesicularis* var. *povalolyticus*, PDH was reported to be a monomeric protein of molecular mass between 65 and 75 kDa. Using pentane-2,4-dione (acetylacetone) as substrate, PDH activity was inhibited by bivalent mercuric and silver ions, NaF and progressively inactivated by increasing concentrations of NaCl.

Mechanisms of 'neutral' β -diketone cleavage

OPH (oxidized PVA hydrolase)

The mechanism whereby oxidized PVA was degraded by β -diketone hydrolase enzymes was further illuminated when the first gene encoding such an activity was isolated. Shimao and co-workers [33] cloned a gene, *pvaB*, from the PVA-degrading bacterium *Pseudomonas* sp. strain VM15C, which formed an operon with *pvaA*, the gene encoding the alcohol dehydrogenase activity that preceded the hydrolytic activity during the biodegradative process. Crucially, the amino acid sequence of one of these oxidized PVA-degrading β -diketone hydrolases was available for the first time. The enzyme, OPH, a protein of 379 amino acids (molecular mass 40 kDa), was shown to contain a GISSG motif from positions 201 to 205, which was coincident with the lipase motif (GXSG) described by Jendrossek et al. [34] in the polyhydroxyalkanoate depolymerase from *Pseudomonas* sp., with which OPH shared 22% sequence similarity. The activity of OPH was assayed *in vitro* using nonane-4,6-dione as substrate, although, interestingly, the enzyme displayed no activity towards pentane-2,4-dione (acetylacetone), as had been described for other oxidized-PVA-cleavage enzymes. PMSF was shown to inhibit the activity completely and, when considered with the consensus lipase motif, this was strongly suggestive of a nucleophilic serine residue in the mechanism of β -diketone cleavage by OPH, although putative residues constituting the rest of a catalytic triad were not identified. Identification of a putative catalytic serine

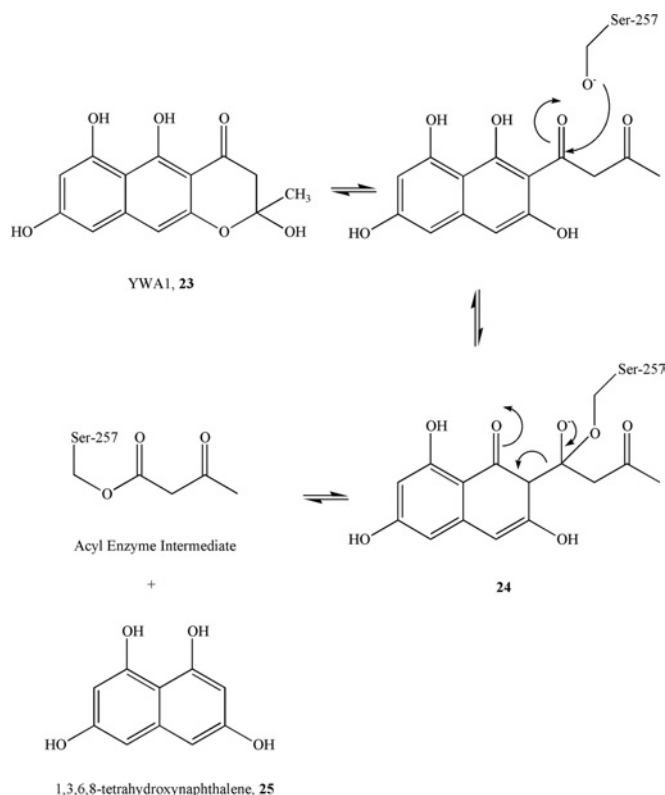


Scheme 8 Suggested mechanism for a serine-triad-type-hydrolase-catalysed cleavage of β -diketones (represented by nonane-4,6-dione)

residue suggests a mechanism for C–C bond cleavage whereby one carbonyl of the substrate is attacked by a nucleophilic serine as part of a catalytic triad to yield a tetrahedral transition state, stabilised perhaps by a suitable oxyanion hole in the active site of OPH. Bond cleavage would result in an acyl-enzyme intermediate and pentan-2-one (Scheme 8). A nucleophilic serine residue had also been identified as the basis of the catalytic mechanism of the enzyme Ayg1p from *Aspergillus fumigatus* [35]. Ayg1p catalyses the processing of the heptaketide YWA1 (**23**) to 1,3,6,8-tetrahydroxynaphthalene (T4HN) (**25**; Scheme 9). The open-chain form of YWA1 features a tetrahydroxynaphthalene nucleus with a pendant acetoacetate moiety. Although it is not a β -diketone hydrolase in the strictest sense of the present review, a nucleophilic serine residue attacks the carbonyl group of the acetoacetate nearest to the ring system, and the resultant oxyanion rearranges to form the β -dioxo species (**24**). This undergoes C–C bond cleavage to form the product T4HN and acetoacetic acid. The evidence that suggests that the serine residue acts as a nucleophile in the mechanism of Ayg1p and this may provide support for the activity of a nucleophilic serine residue in enzymes such as OPH, in contrast with the possibility of a serine residue in a classical catalytic triad acting as a general base for the activation of water, as observed in the mechanism of the C–C-bond-cleaving enzyme MhpC from *Escherichia coli* [36].

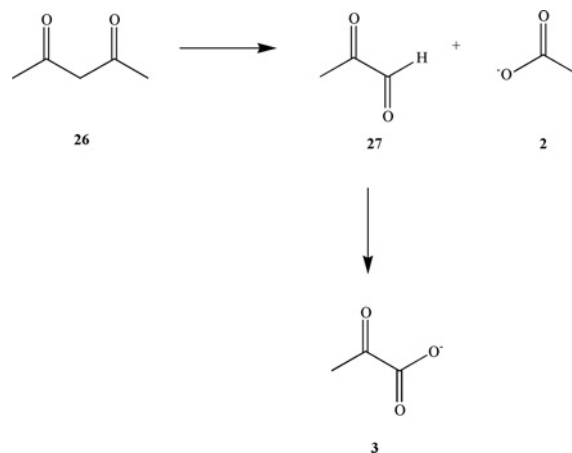
Dke1 (diketone-cleaving enzyme from *Acinetobacter johnsonii*)

The cleavage of the industrially significant β -diketone acetylacetone (pentane-2,4-dione, **26**), to yield acetate and acetone, by the enzyme pentane-2,4-dione hydrolase, has already been described [32]. Straganz and co-workers [37] later isolated by enrichment selection a bacterium, *A. johnsonii*, capable of growth on acetylacetone as sole carbon source. When acetylacetone was incub-



Scheme 9 Mechanism of serine-nucleophile-dependent C–C bond cleavage by the enzyme Ayg1p from *A. fumigatus*

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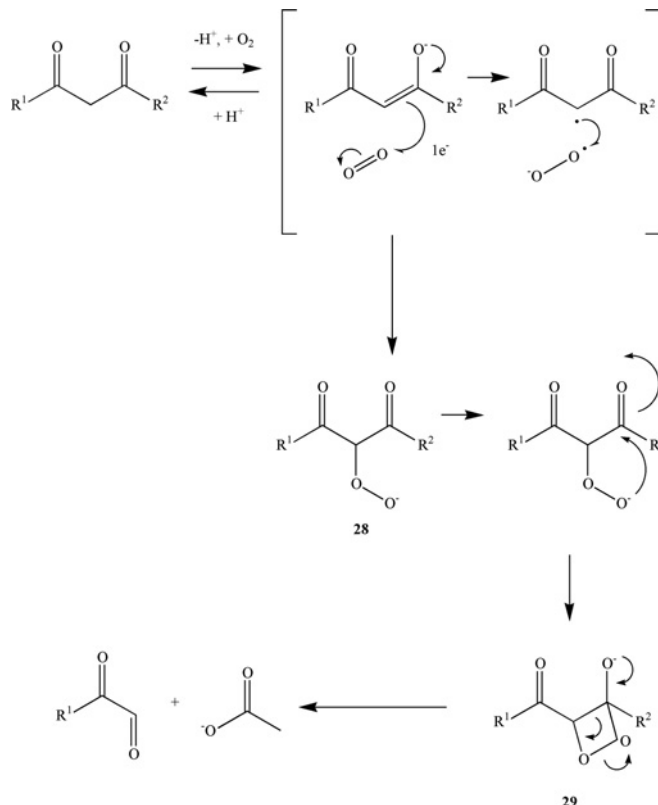


Scheme 10 Cleavage of acetylacetone to glycoaldehyde and acetate by the dioxxygenase Dke1 from *A. johnsonii*

ated *in vitro* with cell extracts of the isolate, the expected product of a β -diketone hydrolase reaction, acetone, was not detected; rather, acetate (**2**) and pyruvate (**3**) were found to be the products. It was found that pyruvate had been evolved from methylglyoxal (**27**) as the initial product of enzymatic C–C bond cleavage (Scheme 10). Surprisingly, therefore, a previously unreported mechanism of β -diketone cleavage had been discovered, and the enzyme that catalysed this process was isolated and characterized.

The enzyme, 'Diketone-cleaving enzyme', designated 'Dke1', was found to be a homogeneous tetrameric metalloenzyme of subunit molecular mass 16 kDa, which is dependent on iron for activity. Other β -diketones, including 2-acetylcyclohexanone and 1-phenylbutane-1,3-dione were accepted as substrates. The gene encoding Dke1 was cloned [38] and shown to encode a protein sequence of 153 amino acids that exhibited little homology with other enzymes in the database. Polarographic and NMR experiments showed that 1 mol of molecular oxygen was consumed for each molecule of acetylacetone cleaved, and hence the C–C-bond-cleavage activity was attributed to a dioxygenase. One iron atom was bound per subunit, and the k_{cat} and K_{m} values for acetylacetone were determined to be 8.5 s^{-1} and $9.1 \text{ }\mu\text{M}$ respectively. It was observed that, for a series of substrates, k_{cat} was largely unaffected, despite large differences in $k_{\text{cat}}/K_{\text{m}}$, suggesting a common rate-limiting step in the cleavage of all substrates accepted by Dke1. Allusion was made in that report [38] to the X-ray-crystallographic structure of Dke1, which shows structural homology with enzymes of the cupin superfamily, a catalytically diverse group of enzymes whose number include aci-reductone dioxygenase [39], cysteine dioxygenase and quercetin dioxygenase, as well as enzymes involved in epoxidation and epimerization reactions [40]. The oxygen-utilizing metalloenzymes of this group of dioxygenases contain redox-active transition metals, which are well known mediators of oxygenating reactions. By interaction of the metal cofactors with triplet oxygen or singlet substrate, it is thought that the reactants can overcome the spin conservation rule [41]. It is likely that, in the case of Dke1, the iron cofactor is not only needed for the appropriate positioning of the substrate, but also provides the electronic environment to render possible a reaction between acetylacetone and oxygen. The structure of Dke1 and ligand complexes thereof will prove an invaluable aid in understanding the mechanism of this dioxygenase in relation to C–C bond cleavage in acetylacetone.

Investigations into the possible mechanism of Dke1 have recently been published [42]. The enzyme was incubated with acetylacetone under an $^{18}\text{O}_2$ atmosphere and the distribution of label analysed by gas chromatography–MS. It was found that, concordant with the suggestion that the enzyme was a dioxygenase, approx 97% of the label was incorporated into acetate and 70% into methylglyoxal, the latter value consistent with some exchange owing to (de)hydration of the aldehyde product. Although three possible mechanisms of bond cleavage by dioxygen were mooted, the authors finally proposed a nucleophilic attack at the electrophilic carbonyl carbon atom by a peroxidate (28) resulting from attack of dioxygen on the enolate form of acetylacetone. Attack of the peroxidate forms a dioxetane (29) that undergoes cleavage to form methylglyoxal and acetate as products (Scheme 11). This conclusion was based on experimental evidence that showed that the product ratio $\text{R}^1\text{CO}_2^-/\text{O}=\text{CHR}^2$, and hence the relative susceptibility of one or other candidate C–C bonds to be cleaved, inverted completely when the substrate R_2 substituent was changed from methyl (electron donating) to trifluoromethyl (electron withdrawing). The bond adjacent to the most electron-deficient carbon atom was cleaved in all cases, thereby supporting a mechanism of nucleophilic attack by an intermediate peroxidate. The determination of the structure of Dke1 in complex with substrates or analogues, and the characterization of the rate-limiting step of reaction, should provide further insight into the detailed mechanism of oxygenative dicarbonyl cleavage.



Scheme 11 Mechanism proposed for Dke1-catalysed cleavage of acetylacetone

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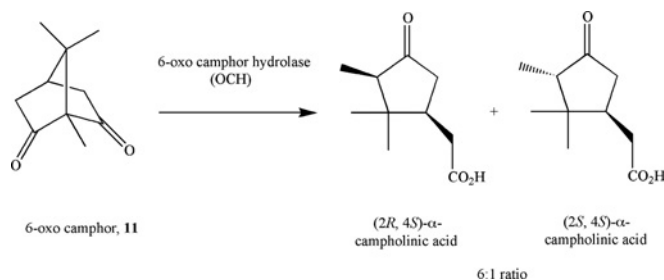
One of these routes, shown in Scheme 4, employed by a *Corynebacterium* species, incorporated the cleavage of the symmetrical β -diketone 6-oxocamphor (11, bornane-2,6-dione, 2,6-diketocamphane) by a ' β -diketone hydrolase' to yield α -campholinic acid (12), a cyclopentanone derivative with two chiral centres that was shown to be optically active.

During a series of investigations focused on enzyme-catalysed stoichiometric asymmetric processes, the early work of Gunsalus and colleagues [25] was revisited, and an enzyme from *Rhodococcus* sp. NCIMB 9784, designated OCH, that was able to catalyse the desymmetrization of 6-oxocamphor *in vitro* to yield a 6:1 mixture of (2*R*,4*S*)- and (2*S*,4*S*)- α -campholinic acid diastereomers with high optical purity, was isolated [43] (Scheme 12). Electrospray MS revealed a subunit molecular mass of approx. 29 kDa, and calibrated gel filtration showed that OCH was a trimer in solution [44]. OCH was shown to hydrolyse a number of monocyclic and bicyclic diketones, but activity appeared to be extremely slow with enolizable β -diketones. However, bicyclo[2.2.1]heptanedione and bicyclo[2.2.2]octanedione were transformed to chiral cyclic oxo acids with high optical purity [43].

The gene encoding OCH was cloned and observed to encode a polypeptide of 257 amino acids [44]. The amino acid sequence displayed some similarity with enzymes of the crotonase superfamily, a group of low-sequence-homology enzymes that catalyse a wide range of different chemical reactions [45]. These include decarboxylation [46], double-bond hydration [47], dehalogenation by the parent member of the superfamily, namely enoyl-CoA hydratase [48] and the isomerization of double bonds in unsaturated fatty acids [49]. In each case, the substrate for each crotonase-catalysed reaction was an acyl-CoA thioester and the reaction

OCH (6-oxocamphor hydrolase from *Rhodococcus* sp.)

In the 1960s, Gunsalus and co-workers [25] were concerned with metabolic routes for the degradation of the monoterpene camphor.



Scheme 12 Stereochemical course of the reaction catalysed by OCH

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chemistries catalysed, though different, were related by the active-site stabilization of an enolate intermediate by an oxyanion hole that was conserved throughout the superfamily. OCH had already been proved to be different from the rest of the crotonase superfamily in two respects: (i) the substrate was not an acyl-CoA thioester and (ii) the conserved oxyanion hole was not present, or present in a different form, since OCH did not possess a GG/AG motif, known to be partially responsible for oxyanion hole formation from crystal structures of known crotonases in complex with substrates or analogues.

The structure of OCH [50] (Figure 5) revealed a cavity in the enzyme monomer, gated by a phenylalanine residue, which featured a number of acid/base residues and an ordered water structure. This cavity was identified as a putative active site and, on this basis, five mutants of OCH, His⁴⁵ → Ala, His¹²² → Ala, His¹⁴⁵ → Ala, Asp¹⁵⁴ → Asn and Glu²⁴⁴ → Gln, were prepared and kinetic constants for each mutant determined [51]. In each case the second-order rate constants were substantially lowered, but the effect was most notable for the His¹⁴⁵ → Ala mutant, where k_{cat} was very low and the C–C-bond-cleavage activity, as measured by the disappearance of substrate using a UV spectrophotometric assay, barely measurable, suggesting a crucial role in catalysis. The low- k_{cat} -plus-low- K_{m} mutant, His¹²² → Ala, was selected for co-crystallization experiments with the substrate 6-oxocamphor,

as it was thought that this would present the best opportunity for trapping the substrate ligand in the active site.

The structure of the His¹²² → Ala mutant that had been soaked with the natural substrate 6-oxocamphor was solved to 1.9 Å (0.19 nm) resolution [51]. However, instead of the substrate being bound in the active site, the product, α -campholinic acid was observed. In addition, the diastereomer that was bound was not the (2*R*,4*S*) *cis* form that predominates in the product extracted from *in vitro* reactions conducted using pure wild-type OCH, but rather the (2*S*,4*S*) *trans* form that constitutes the minor product. It was reasoned that this diastereomer would tautomerize to the more thermodynamically stable predominant isomer when released from the confines of the active site.

The presence of the ligand in the active site nevertheless revealed several interesting interactions with active site residues that could make a contribution either to mechanism or prochiral selectivity (Figure 6). One carboxylate oxygen atom of the pendant acetate group at position 4 was hydrogen-bonded to His¹⁴⁵, which in turn forms a dyad with Asp¹⁵⁴. As a water molecule is present in approximately the same site in the native structure, this provides good evidence for the base-catalysed activation of a water molecule for nucleophilic attack at the carbonyl group on the *pro-S* face of the 6-oxocamphor molecule. In addition, the carbonyl group of the *pro-R* face of 6-oxocamphor, now the carbonyl group of the forming cyclopentanone ring, was hydrogen-bonded to Trp⁴⁰, and the forming flattened five-membered ring stacked against Phe⁸² (Figure 6). These appeared to be among the significant factors contributing to prochiral facial selectivity in OCH, the only β -diketone cleavage enzyme for which a chiral selective transformation has been reported to date.

A proposed mechanism is shown in Scheme 13. A water molecule is activated by His¹⁴⁵ for nucleophilic attack of hydroxide at the *pro-S* face of the symmetrical substrate. This would result in a tetrahedral oxyanion (**30**) that would require stabilization within the active site. The oxyanion would rearrange to yield enolate (**31**), held by Trp⁴⁰ and His¹²², which is then protonated to yield the final (2*S*,4*S*) product, which tautomerizes outside the confines of the active site to yield the thermodynamically stable mixture of (2*R*,4*S*) and (2*S*,4*S*) diastereomers in a 6:1 ratio. Further knowledge of the active-site topology and mechanism

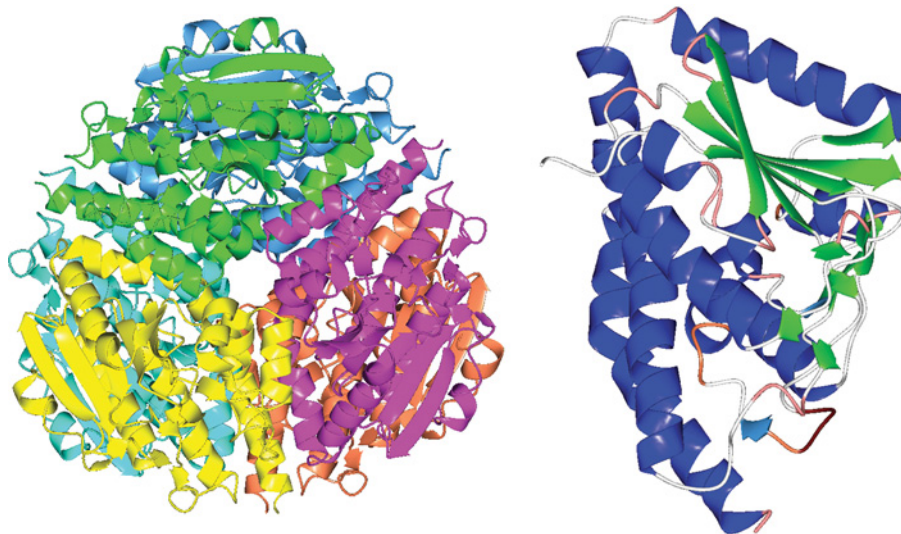


Figure 5 Structure of OCH

OCH is a hexamer (left) of 29 kDa subunit monomers (right).

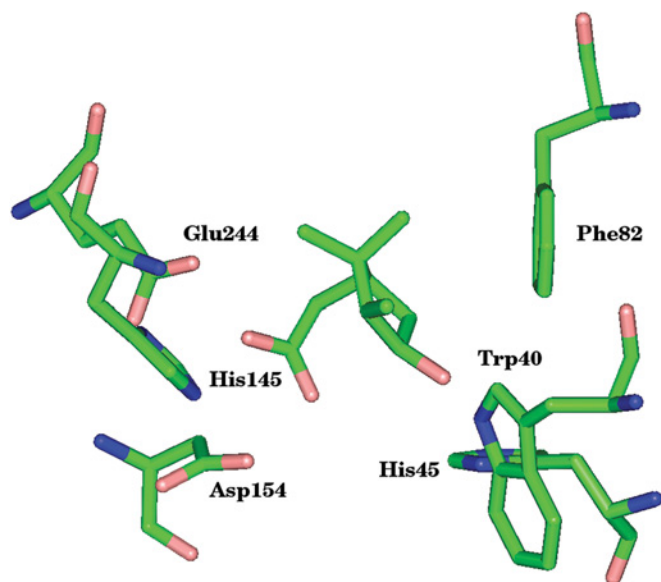
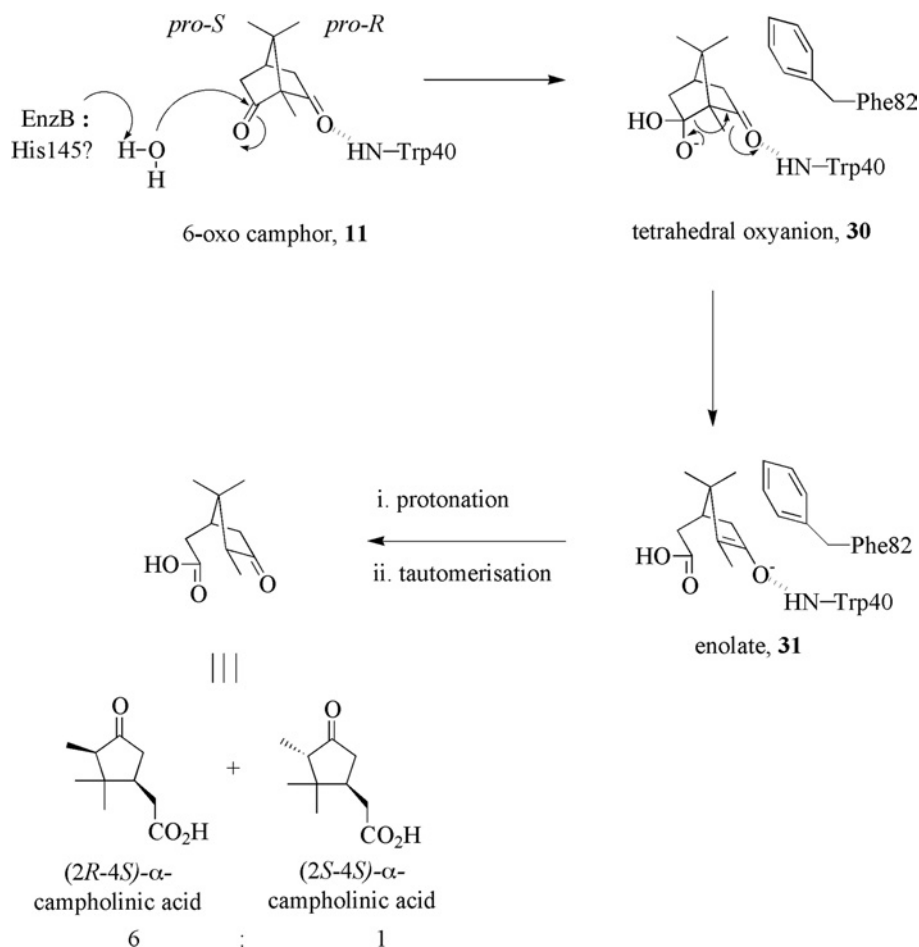


Figure 6 Active site of OCH showing interaction of active-site residues with (2*S*,4*S*)- α -campholinic acid

of OCH could aid in the engineering of isoenzymes capable of opposite prochiral selectivities, in order to furnish an series of products enantiocomplementary to those currently available.

SUMMARY

The mechanisms adopted by Nature for the cleavage of the β -diketone function are remarkably diverse, especially considering the small number of enzymes described thus far that are capable of this transformation. It is possible that this diversity has arisen to accommodate the cleavage of C–C bonds of different susceptibility. The mechanism of FAH is similar to that of OCH but, whereas a metal ion is needed to aid catalysis by the former enzyme, the strain inherent in the OCH substrate may offset the need for additional activation of a water-derived hydroxide in the latter. The contrasting mechanisms of aliphatic β -diketone cleavage in OPH and Dke1 may reflect differing oxygen availabilities in the bacterial environment. However, it is clear that, in common with natural mechanisms of proteolysis, it has been necessary for Nature to recruit different protein sequences, three-dimensional folds and mechanistic chemistry to effect the cleavage of C–C bonds in β -diketones for the purposes of detoxification and catabolic processes to secure metabolic energy. The results described above illustrate how these different mechanisms may be



Scheme 13 Mechanism proposed for cleavage of the C–C bond in 6-oxocamphor by OCH

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exploited for the greater understanding of these enzymes and their potential uses in medicinal chemistry, bioremediation and preparative biocatalysis.

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REFERENCES

- 1 Fargeas, V., Baalouch, M., Metay, E., Baffreau, J., Ménard, D., Gosselin, P., Bergé, J.-P., Barthelemeuf, C. and Lebreton, J. (2004) New access to 1,3-diketones from aldehydes. *Tetrahedron* **60**, 10359–10364
- 2 Christoffers, J., Oertling, H., Fischer, P. and Frey, W. (2003) Studies on the α -acetylation of δ -valerolactone and ϵ -caprolactone. *Tetrahedron* **59**, 3769–3778
- 3 Katritzky, A. R., Wang, Z., Wang, M., Wilkerson, C. R., Hall, D. and Akhmedov, N. G. (2004) Preparation of β -keto esters and β -diketones by C-acylation/deacetylation of acetoacetic esters and acetyl ketones with 1-acylbenzotriazoles. *J. Org. Chem.* **69**, 6617–6622
- 4 Kumar, D. and Singh, S. P. (2004) Structural revision in pyrazole chemistry. *Heterocycles* **63**, 145–173
- 5 Garnovskii, A. D., Kharixov, B. I., Blanco, L. M., Garnovskii, D. A., Burlov, A. S., Vasilchenko, I. S. and Bondarenko, G. I. (1999) Some aspects of competitive coordination of β -diketones and nitrogen-containing ligands. *J. Coord. Chem.* **46**, 365–375
- 6 Diana, G. D., Carabateas, P. M., Johnson, R. E., Williams, G. L., Pancic, F. and Collins, J. C. (1978) Antiviral activity of some β -diketones. 4. Benzyl diketones. *In vitro* activity against both RNA and DNA viruses. *J. Med. Chem.* **21**, 889–894
- 7 Pokorny, D., Steiner, W. and Ribbons, D. W. (1997) β -Ketolases – forgotten hydrolytic enzymes? *Trends Biotechnol.* **15**, 291–296
- 8 Grogan, G. (2002) β -Diketone hydrolases. *J. Mol. Catal. B Enzym.* **19**, 73–82
- 9 Reference deleted
- 10 Babbitt, P. C. (2003) Definitions of enzyme function for the structural genomic era. *Curr. Opin. Chem. Biol.* **7**, 230–237
- 11 Nishizuka, Y., Ichiyama, A., Nakamura, S. and Hayaishi, O. (1961) A new metabolic pathway of catechol. *J. Biol. Chem.* **237**, 268–270
- 12 Davey, J. F. and Ribbons, D. W. (1975) Metabolism of resorcinolic compounds by bacteria. Purification and properties of acetylpyruvate hydrolase from *Pseudomonas putida* 01. *J. Biol. Chem.* **250**, 3826–3830
- 13 Pokorny, D., Brecker, L., Pogorevc, M., Steiner, W., Griengl, H., Kappe, T. and Ribbons, D. W. (1999) Proton-nuclear magnetic resonance analyses of the substrate specificity of a β -ketolase from *Pseudomonas putida*. *J. Bacteriol.* **181**, 5051–5059
- 14 Mitchell, G. A., Lambert, M. and Tanguay, R. M. (1995) Hypertyrosinemia. In *The Metabolic and Molecular Basis of Inherited Disease*, 7th edn (Scriver, C. R., Beaudet, A. L., Sly, W. S. and Valle, D., eds.), pp. 1076–1107, McGraw-Hill, New York
- 15 Knox, W. E. and Edwards, S. W. (1955) The properties of maleylacetoacetate, the initial product of homogentisate oxidation in liver. *J. Biol. Chem.* **216**, 489–498
- 16 Chapman, P. J. and Dagley, S. (1962) Oxidation of homogentisic acid by cell-free extracts of a vibrio. *J. Gen. Microbiol.* **28**, 251–256
- 17 Saing, H. H., Sim, S. S., Mahuran, D. J. and Schmidt, D. E. (1972) Purification and properties of a diketo acid hydrolase from beef liver. *Biochemistry* **11**, 2098–2102
- 18 Mahuran, D. J., Angus, R. H., Braun, C. V., Sim, S. S. and Schmidt, D. E. (1977) Characterisation and substrate specificity of fumarylacetoacetate fumarylhydrolase. *Can. J. Biochem.* **55**, 1–8
- 19 Timm, D. E., Mueller, H. A., Bhanumoorthy, P., Harp, J. M. and Bunick, G. J. (1999) Crystal structure and mechanism of a carbon–carbon bond hydrolase. *Structure* **7**, 1023–1033
- 20 Bateman, R. L., Bhanumoorthy, P., Witte, J. F., McClard, R. W., Grompe, M. and Timm, D. E. (2001) Mechanistic inferences from the crystal structure of fumarylacetoacetate hydrolase with a bound phosphorus-based inhibitor. *J. Biol. Chem.* **276**, 15284–15291
- 21 Hopper, D. J., Chapman, P. J. and Dagley, S. (1971) The enzymic degradation of alkyl-substituted gentisates, maleates and malates. *Biochem. J.* **122**, 29–40
- 22 Poh, C. L. and Bayly, R. C. (1980) Evidence for isofunctional enzymes used in *m*-cresol and 2,5-xenol degradation via the gentisate pathway in *Pseudomonas alcaligenes*. *J. Bacteriol.* **143**, 59–69
- 23 Bayly, R. C., Chapman, P. J., Dagley, S. and Berardino, D. D. (1980) Purification and some properties of maleylpyruvate and fumarylpyruvate hydrolase from *Pseudomonas alcaligenes*. *J. Bacteriol.* **143**, 70–77
- 24 Zhou, N.-Y., Fuenmayor, S. L. and Williams, P. A. (2001) *nag* Genes of *Ralstonia* (formerly *Pseudomonas*) sp. strain U2 encoding enzymes for gentisate catabolism. *J. Bacteriol.* **183**, 700–708
- 25 Chapman, P. J., Meerman, G., Gunsalus, I. C., Srinivasan, R. and Rinehart, K. (1966) New acyclic acid metabolite in camphor oxidation. *J. Am. Chem. Soc.* **88**, 618
- 26 Dangel, W., Tschach, A. and Fuchs, G. (1989) Enzyme reactions involved in anaerobic cyclohexanol metabolism by a denitrifying *Pseudomonas* species. *Arch. Microbiol.* **152**, 273–279
- 27 Bartholomew, B. A., Smith, M. J., Trudgill, P. W. and Hopper, D. J. (1996) Atropine metabolism by *Pseudomonas* sp. strain AT3: evidence for nortropine as an intermediate in tropine breakdown and reactions leading to succinate. *Appl. Environ. Microbiol.* **62**, 3245–3250
- 28 Sakai, K., Morita, M., Hamada, N. and Watanabe, Y. (1981) Studies on the poly(vinyl alcohol) degrading enzyme. 3. Purification and properties of oxidized poly(vinyl alcohol)-degrading enzyme. *Agric. Biol. Chem.* **45**, 63–71
- 29 Sakai, K., Hamada, N. and Watanabe, Y. (1985) Studies on the poly(vinyl alcohol) degrading enzyme. 5. Purification and properties of oxidized poly(vinyl alcohol) hydrolase with an acidic isoelectric point. *Agric. Biol. Chem.* **49**, 827–833
- 30 Sakai, K., Hamada, N. and Watanabe, Y. (1985) A new enzyme, β -diketone hydrolase: a component of a poly(vinyl alcohol)-degrading enzyme preparation. *Agric. Biol. Chem.* **49**, 1901–1902
- 31 Sakai, K., Hamada, N. and Watanabe, Y. (1986) Degradation mechanism of poly(vinyl alcohol) by successive reactions of secondary alcohol oxidase and β -diketone hydrolase from *Pseudomonas* sp. *Agric. Biol. Chem.* **50**, 989–996
- 32 Kawagoshi, Y. and Fujita, M. (1998) Purification and properties of the poly(vinyl alcohol) degrading enzyme 2,4-pentanedione hydrolase obtained from *Pseudomonas vesicularis* var. *povalolyticus*. *PH. World J. Microbiol. Biotechnol.* **14**, 95–100
- 33 Shimao, M., Tamogami, T., Kishida, S. and Harayama, S. (2000) The gene *pvaB* encodes oxidized poly(vinyl alcohol) hydrolase of *Pseudomonas* sp. strain VM15C and forms an operon with the poly(vinyl alcohol) dehydrogenase gene *pvaA*. *Microbiology* **146**, 649–657
- 34 Jendrosseck, D., Müller, B. and Schlegel, H. G. (1993) Cloning and characterization of the poly(hydroxyalkanoic acid)-depolymerase gene locus, *PHAZ1*, of *Pseudomonas lemoignei* and its gene product. *Eur. J. Biochem.* **218**, 701–710
- 35 Fujii, I., Yasuoka, Y., Tsai, H.-F., Chang, Y. C., Kwon-Chung, K. J. and Ebizuka, E. (2004) Hydrolytic polyketide shortening by Agy1p, a novel enzyme in fungal melanin biosynthesis. *J. Biol. Chem.* **279**, 44613–44620
- 36 Fleming, S. M., Robertson, T. A., Langley, G. J. and Bugg, T. D. H. (2000) Catalytic mechanism of a C–C hydrolase enzyme: Evidence for a gem-diol intermediate, not an acyl enzyme. *Biochemistry* **39**, 1522–1531
- 37 Straganz, G., Brecker, L., Weber, H.-J., Steiner, W. and Ribbons, D. W. (2002) A novel β -diketone-cleaving enzyme from *Acinetobacter johnsonii*: acetylacetone 2,3-oxygenase. *Biochem. Biophys. Res. Commun.* **297**, 232–236
- 38 Straganz, G. D., Glieder, A., Brecker, L., Ribbons, D. W. and Steiner, W. (2003) Acetylacetone-cleaving enzyme Dke1: a novel C–C bond cleaving enzyme from *Acinetobacter johnsonii*. *Biochem. J.* **369**, 573–581
- 39 Pochapsky, T. C., Pochapsky, S. S., Ju, T., Mo, H., Al-Mjeni, F. and Maroney, M. J. (2002) Modeling and experiment yields the structure of acireductone dioxygenase from *Klebsiella pneumoniae*. *Nat. Struct. Biol.* **9**, 966–972
- 40 Dunwell, J. M., Purvis, A. and Khuri, S. (2004) Cupins: the most functionally diverse superfamily? *Phytochemistry* **65**, 7–17
- 41 Costas, M., Mehn, M. P., Jensen, M. P. and Que, Jr, L. (2004) Dioxygen activation at mononuclear nonheme iron active sites: enzymes, models and intermediates. *Chem. Rev.* **104**, 939–986
- 42 Straganz, G. D., Hofer, H., Steiner, W. and Nidetzky, B. (2004) Electronic substituent effects on the cleavage specificity of a non-heme Fe²⁺-dependent β -diketone dioxygenase and their mechanistic implications. *J. Am. Chem. Soc.* **126**, 12202–12203
- 43 Grogan, G., Graf, J., Jones, A., Parsons, S., Turner, N. J. and Flitsch, S. L. (2001) An asymmetric enzyme-catalyzed retro-Claisen reaction for the desymmetrisation of cyclic β -diketones. *Angew. Chem. Int. Ed. Engl.* **40**, 1111–1114
- 44 Grogan, G., Roberts, G. A., Bougioukou, D., Turner, N. J. and Flitsch, S. L. (2001) The desymmetrisation of bicyclic β -diketones by an enzymatic retro-Claisen reaction. A new reaction of the crotonase superfamily. *J. Biol. Chem.* **275**, 12565–12572
- 45 Holden, H. M., Benning, M. M., Haller, T. and Gerlt, J. A. (2001) The crotonase superfamily: Divergently related enzymes that catalyse different reactions involving acyl coenzyme A thioesters. *Acc. Chem. Res.* **34**, 145–157
- 46 Benning, M. M., Haller, T., Gerlt, J. A. and Holden, H. M. (2000) New reactions in the crotonase superfamily: Structure of methylmalonyl-CoA decarboxylase from *Escherichia coli*. *Biochemistry* **39**, 4630–4639
- 47 Engel, C. K., Mathieu, M., Zeelen, J. P., Hiltunen, J. K. and Wierenga, R. K. (1996) Crystal structure of enoyl-coenzyme A (CoA) hydratase at 2.5 angstroms resolution: a spiral fold defines the Co-A binding pocket. *EMBO J.* **15**, 5135–5145

-
- 48 Benning, M. M., Taylor, K. L., Liu, R.-Q., Yang, G., Xiang, H., Wesenberg, G., Dunaway-Mariano, D. and Holden, H. M. (1996) Structure of 4-chlorobenzoyl coenzyme A dehalogenase determined to 1.8 Å resolution: An enzyme catalyst generated by adaptive mutation. *Biochemistry* **35**, 8103–8109
- 49 Modis, Y., Filippula, S. A., Novikov, D. K., Norledge, B., Hiltunen, J. K. and Wierenga, R. K. (1998) The crystal structure of dienoyl-CoA isomerase at 1.5 Å resolution reveals the importance of aspartate and glutamate sidechains for catalysis. *Structure* **15**, 957–970
- 50 Whittingham, J. L., Turkenburg, J. P., Verma, C. S., Walsh, M. A. and Grogan, G. (2003) The 2 Å crystal structure of 6-oxocamphor hydrolase. New structural diversity in the crotonase superfamily. *J. Biol. Chem.* **278**, 1744–1750
- 51 Leonard, P. M. and Grogan, G. (2004) Structure of 6-oxo camphor hydrolase H122A mutant bound to its natural product (2S, 4S)- α -campholinic acid. Mutant structure suggests an atypical mode of transition state binding for a crotonase homolog. *J. Biol. Chem.* **279**, 31312–31317
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